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Cytokine antibody array analysis in brain and periphery of scrapie-infected Tg338 mice

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ABSTRACT

Scrapie is a prion-associated transmissible spongiform encephalopathy (TSE) of sheep and goats, and frequently serves as a comparative model for other prion diseases, such as chronic wasting disease and bovine spongiform encephalopathy. TSEs are unique neurologic disorders that do not appear to be accompanied by robust systemic immunologic responses. mRNA data suggest that cytokines are involved in scrapie progression. In this study, brain tissue, mesenteric lymph nodes, splenic tissue and serum from ovinized mice were screened for 62 cytokine and cytokine-related proteins at pre-clinical and clinical points of infection. Expression patterns were compared to brain histology and clinical presentation. Increased cytokine expression in the brain and periphery were noted in scrapie-positive animals before histologic changes or clinical signs were evident. Of the 62 proteins examined, only IL-10 and TIMP-1 were consistently expressed at increased levels in the serum throughout infection. These cytokines could suggest future targets for biomarkers of infection and may, as well, provide insight into the biologic dynamics of prion-associated neurologic diseases.

1. Introduction

The transmissible spongiform encephalopathies (TSEs) are inevitably fatal neurodegenerative disorders [1]. Human TSEs include a diverse group of iatrogenic, sporadic, familial and acquired disorders, including Creutzfeldt-Jakob Disease and Kuru. TSEs of livestock include chronic wasting disease (CWD) of elk, mule deer, white-tailed deer and moose, bovine spongiform encephalopathy (BSE) of cattle, and scrapie disease of sheep and goats [2]. A disease-associated isoform of the prion protein (e.g., PrP^{Sc}) is thought to be the etiologic agent of the TSEs [3]. The cellular isoform (PrP^{c}) is expressed in brain and peripheral tissues of mammals, although its function is still not fully understood. Disease occurs when PrP^{c} is induced to misfold into PrP^{Sc}, resulting in a conformation with increased beta-sheets. Histologically, TSEs ultimately present with neuronal apoptosis, astrogliosis and spongiform degeneration or neuropil vacuolization without distinct inflammatory cell accumulation [4]. The mechanisms by which accumulation of PrP^{Sc} results in neurodegenerative changes are not entirely understood. Clinically, TSEs present with progressive neurodegenerative signs, often including ataxia, severe disorientation or behavioral changes, and myoclonic twitching. Disease incubation is long and clinical signs are usually not obvious until the terminal stages of infection.

TSEs are unique neurologic disorders in which measurable systemic immune responses do not seem to be evoked by the causative agent [1,5]. Attempts to demonstrate antibody to the scrapie agent by neutralization, complement fixation, immunoprecipitation and immunofluorescence

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have been unsuccessful, and scrapie-positive immunodeficient mice depleted of T-lymphocytes show disease course similar to that of immunocompetent scrapie positive animals [6]. Brain inflammation has been noted in conjunction with TSEs, but in some model systems appears to consist primarily of astrocyte and microglia activation [4], suggesting that these resident central nervous system cells are responsible for increased cytokine expression and subsequent neurodegeneration [5,7]. Increases in cytokine gene expression in murine models of scrapie infection have been reported. Campbell [7] reported a marked increase in tumor necrosis factor alpha (TNFα), Interleukin-1 alpha (IL-1α) and IL-1 beta (IL-1β) mRNA in the terminal stages of murine scrapie. Similar increases in mRNA for pro-inflammatory cytokines in the brain have been found by other groups in several scrapie murine models [8,9]. Gene expression profiling technologies that allow large panels of genes to be analyzed at one time have allowed for a large amount of information to be collected on cytokine gene expression levels throughout the course of scrapie infection [10]. However, protein levels do not always correlate with mRNA expression levels and protein level analyses may be useful for identification of diagnostic markers and treatment targets [11]. Immunostaining procedures can be employed to explore protein expression, but often the process lacks quantification and is laborious since only one or a few proteins are probed at one time. Relatively new antibody array technology has improved upon this situation and made simultaneous measurement of a large panel of proteins possible. Such arrays could be informative in the ongoing study of the role of cytokines in prion disease pathogenesis.

In this study, brain tissue, mesenteric lymph nodes, splenic tissue and serum from ovinized transgenic mice infected with scrapie (inoculated intracerebrally with scrapie-positive sheep brain) were screened for 62 different cytokine and cytokine-related proteins at pre-clinical and clinical time points during infection. Protein expression assays were used to determine whether cytokine and cytokine-related protein levels in the brain or periphery increased or decreased during infection and whether such changes were associated with brain histopathology and clinical presentation. A global analysis of cytokine protein expression at preclinical and clinical scrapie time points in brain and peripheral tissues could contribute critical information to the pathogenesis of prion infection and/or identify candidate biomarkers for infection or progression of scrapie or similar agents.

2. Materials and methods

2.1. Animals

Thirty-five 8–12 week old homozygous Tg338 mice transgenic for the sheep prion gene (*PRNP*) VRQ allele on a mouse PrP−/− background were used for these studies. The Tg338 mice were maintained and bred at the University of Washington. The mice originated from the laboratory of H. Laude (Jouy-en-Josas, France) and were developed as previously described [12,13]. PrP expression level in the brain of Tg338 animals is 8–10 fold that in sheep [12]. All treatments and procedures were approved by the University of Washington Institutional Animal Care and Use Committee. Mice were housed under Biosafety Level-2 (BSL-2) conditions in a specific pathogen-free facility with a 12:12-h light:dark cycle at room temperatures between 20 and 23 °C. Animals were fed irradiated chow (Picolab Rodent Diet 20 number 5053, PMI Nutrition) and provided autoclaved, acidified water. Sentinel mice were tested quarterly and determined negative for endo- and ectoparasites, mouse hepatitis virus, mouse parvovirus, and rotavirus, and annually for Mycoplasma pulmonis, pneumonia virus of mice, reovirus 3, Sendai virus, and Theiler murine encephalomyelitis virus.

Twenty-one mice were anesthetized with a combination of ketamine and xylazine and were inoculated intracerebrally (i.c.) with 30 μL of 10% (w/v) homogenate of brain from a scrapie-positive sheep. Seven Tg338 mice were inoculated i.c. with 30 μL of 10% (w/v) homogenate of scrapie-negative sheep brain. Seven uninoculated Tg338 mice were used as additional controls for the appearance of clinical signs in inoculated mice.

2.2. Cytokine assays

2.2.1. Tissue lysate and serum preparation

Mice infected with scrapie-positive sheep brain homogenate were euthanized by cervical dislocation at one of three time points (7 per group): two preclinical time points (100 and 160 days post inoculation) and one clinical time point (190–268 days post inoculation, referred to as ≥190 days post inoculation). Tg338 mice inoculated with scrapie-negative sheep brain acted as negative controls, and tissues were collected at time points similar to those of clinical scrapie-positive sheep brain inoculated animals. Blood collected by cardiac puncture was centrifuged and the serum stored at −80 °C. The brain, spleen, and mesenteric lymph nodes were harvested from euthanized animals. A portion of brain was saved in 10% neutral buffered formalin for histological evaluation and PET blotting. Western blots were performed on pooled brain tissues that had been frozen at −80 °C and splenic tissue from individual animals stored similarly. Lysis buffer (Raybiotech, Inc) containing proteinase inhibitor (ThermoScientific) was added at a dose of 500μL/10 mg tissue to remaining tissues collected at the time of euthanasia. Tissues were homogenized (Ultrasonic Cell Disrupter, XL-2000, Misonix, Inc.) and centrifuged at 10,000 × *g* for 5 min. The supernatant was held at −80 °C.

2.2.2. Assay protocol

Raybio® Mouse Cytokine Antibody Arrays G Series 3 were performed according to the manufacturer's protocol (Raybiotech, Inc.) on tissues from individual animals. This protocol involved the use of a bicinchoninic acid (BCA) protein assay (Thermo Scientific) to measure and establish 200 μg of protein for each tissue lysate sample. Lysates were subsequently diluted 10-fold with blocking buffer. Serum samples were diluted 5-fold with blocking buffer. Each array slide contained internal controls, provided in the kit, to normalize data. Array slides were incubated for 30 min with blocking buffer and decanted. 100 μL of diluted

protein or serum sample were added to each assay well and incubated overnight at 4 °C. Samples were decanted and the plate was washed 3 times with 150 μL of provided wash buffer I at room temperature. The array slides were then covered with wash buffer I and incubated for 20 min. This wash was repeated with wash buffer II. 300 μL of biotin-conjugated antibodies were added to each assay well. Assay slides were incubated overnight at 4 °C, then washed again with wash buffer I and II. 70 μL of provided fluorescent dye-conjugated streptavidin were added to each well, and the assay slides were covered with adhesive film and aluminum foil to avoid light exposure. The assay slides were incubated at room temperature for 2 h and then washed with wash buffer I. The assay slides were then placed in a 50 mL centrifuge tube and washed with wash buffer I and II, rinsed with distilled water and centrifuged at 1000 rpm for 3 min to remove water droplets. The slides were re-covered in aluminum foil and stored at −20 °C until they were shipped to Raybiotech, Inc. for laser scanner detection.

2.3. Western blots

2.3.1. Brain

Pooled brain tissue from Tg338 mice inoculated with scrapie-positive sheep homogenate was mixed with lysis buffer (0.5% NP-40 and 0.5% sodium deoxycholate in 10 mM Tris buffer, pH 7.5) to form a 10% homogenate. Proteinase K (PK) digestion was performed with PK at 50 μg/mL at 56 °C for 40 min, with inactivation at 99 °C for 10 min. Brain tissue from Tg338 mice inoculated with scrapie-positive or scrapie-negative sheep homogenate were run without PK digestion as additional controls. Western blot samples were denatured, run on a 12% BisTris gel in MOPS SDS running buffer (Invitrogen) at 200 V for 1 h, and transferred to a methanol-soaked PVDF membrane in MOPS transfer buffer (Invitrogen) at 200 mA for 1 h. Membranes were dried and blocked for 1 h in Tris/casein buffer (Roche) with 0.1% Tween 20. Membrane transfer, blocking, and all subsequent steps were done at room temperature. Membranes were probed overnight with 1.2 μg primary mouse mAb F99/97.6.1 (K. O'Rourke), which recognizes prion epitope QYQRES [14], followed by biotinylated goat anti-mouse secondary antibody (Southern Biotech) and enhanced chemiluminescence (GE Healthcare) substrate. Western detection was performed with a commercial apparatus (AlphaImager; Alpha Innotech Corporation).

2.3.2. Spleen

Spleen homogenates (2%, w/v) from Tg338 mice 100, 160 and ≥190 d.p.i with scrapie-positive sheep brain homogenate were analyzed for relative PrP^{Sc} content. Reference brain homogenate (10%, w/v) from clinically affected Tg338 mouse inoculated with scrapie-positive sheep homogenate and a spleen homogenate (2%, w/v) from a Tg338 mouse inoculated with scrapie-negative sheep were also included. PrP^{Sc} was enriched from direct tissue lysis buffer by phosphotungstic acid (PTA) precipitation as described [15] with minor modifications. Briefly, 400 μL direct lysis buffer (0.5% NP-40, 0.5% sodium deoxycholate, 10 mM Tris–HCl, pH 7.5) and 50 μL 10× trypsin was added to 100 μL homogenate and incubated at 37 °C overnight. Brain homogenates were subsequently incubated at 37 °C for 15 min with 500 μL of 4% sarkosyl in phosphate buffered saline (pH 7.4) and then at 37 °C for 45 min with DNase 1 (100 μg/mL). The supernatant (1000 × *g* for 6 min) of each homogenate was incubated at 37 °C for 90 min with 4% PTA (81 μL added to brain homogenate, 41 μL added to spleen homogenate; final concentration 0.3% PTA) after which the pellet (21,000 × *g* for 30 min) was mixed and incubated at 37 °C for 60 min with 200 μL of direct lysis buffer containing PK (200 μg/mL for brain homogenate; 1000 μg/mL for spleen homogenate). The final pellet (21,000 × *g* for 30 min) of each homogenate was dissolved in 10 μL of water. For western blotting, 10 μL of each PTA-precipitated homogenate sample was boiled for 10 min with 13 μL 2× NuPAGE buffer (Invitrogen, Carlsbad, CA) and 2.3 μL DTT (Invitrogen, Carlsbad, CA). Precipitated proteins were resolved on 12% SDS polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Analysis was performed as previously described [16] using monoclonal antibody F99/97.6.1 (binding residues 220–225 of the ovine prion protein; 3.5 μg/mL) and goat anti-mouse IgG-HRPO (SouthernBiotech, Birmingham, AL). Protein bands were visualized using chemiluminescence (GE HealthCare Amersham, UK), Kodak Bio-Max Light film and a Kodak X-OMatdeveloper. Developed films were digitally imaged and relative three-band densities determined (AlphaEase, v4.0; Alpha innotech, San Leandro, CA).

2.4. Paraffin-embedded tissue (PET) blot methods

The PET blot was performed on tissues from individual animals as described previously by Schulz-Schaeffer and others [17,18] with some modifications. Briefly, 3 μm sections of paraffin-embedded tissues were cut, collected onto 0.45 μm nitrocellulose membranes (GE Healthcare), dried for 1 h and then baked for 1 h at 60 °C. The membranes were de-paraffininzed by immersion in xylene 2× (each 5 min), rinsed in isopropanol 2× (each 5 min), and then rehydrated in water with 0.1% Tween-20 for 10 min. The membranes were dried at room temperature. After wetting membranes with TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.0) for 10 min, they were incubated overnight at 55 °C in proteinase K buffer (10 mM Tris–HCl, pH 7.8, 100 mM NaCl) containing 200 μg/mL proteinase K (Roche). After washing the membranes with TBST, sections were treated at 55 °C with 3 M guanidine isothiocyanate for 15 min. The membranes were washed 3× in TBST. Immunodetection was carried out after pre-incubation for 1 h in blocking solution (nonfat milk powder diluted to 0.2% (w/v) in TBST), then incubating the membranes with primary antibody (mAb F99/97.6.1, 3.5 μg/mL diluted in blocking buffer) for 3 h at room temperature. After 3× washing in TBST, an alkaline phosphatase-coupled goat anti-mouse antibody (4751-1806 KPL) diluted 1:1000 in TBST was applied for 45 min. After 4× washing with TBST, the pH was adjusted by rinsing the blots twice with NTM (100 mM

Tris–HCl, pH 9.5, 100 mM NaCl and 50 mM MgCl2). Finally BCIP/NBT (KPL) was used to visualize the antibody reaction products, which were dark blue deposits. PET blot membranes were evaluated and digitally imaged using a dissection microscope. For comparative purposes, images were captured as uncompressed tiff files using uniform lighting, focal distance, magnification, and camera white balance and exposure time. Images were processed using the ImageJ-based open source processing package, Fiji (http://pacific.mpi-cbg.de/) [19].

2.5. Histology

Paraffin-embedded, formalin-fixed brain tissues underwent formic acid treatment and standard processing. Tissues were sectioned at 5 μm, stained with hematoxylin and eosin stain (H&E), and evaluated with particular attention to the obex. The obex is a target area of the central nervous system for scrapie diagnosis [20,21]. Obex sections were assigned vacuolization scores based on criteria described by Bruce [22]. Such scoring is used to denote non-affected (score of 0–1), mildly to moderately affected (score of 2–3) and severely affected animals (score of 4–5). Immunohistochemistry for glial fibrillar acidic protein (GFAP) was performed as previously described [23] on obex sections comparable to those stained with H&E and sections were evaluated for gliosis and photographed for comparison.

2.6. Assay data organization

In order to compare results for assays performed at different times, signal intensities for each cytokine were divided by the positive control signal value. A positive control in the form of biotinylated protein was run with each individual assay and was the manufacturer's suggested method of normalization. Normalized data from Tg338 mice infected with scrapie-positive sheep brain was compared to normalized data from Tg338 mice infected with scrapie-negative sheep brain in order to determine if cytokine levels in scrapie-positive animals were increased or decreased over scrapie-negative controls.

2.7. Statistical analysis

All cytokine data were tabulated. Further statistical tests were performed on TIMP-1 and IL-10, the only cytokines tested that had increased expression over controls in the serum at all time points. Values from mice infected with scrapie-positive homogente were compared to scrapie-negative brain inoculated control mice. The unpaired two-tailed Student's *t*-test was used for analysis. Welch's *t*-test was used when groups had unequal variances. Differences were considered significant at $p < 0.05$. GraphPad Prism 5 for Windows, Version 5.02, was used for analysis. Similar tests were also performed on spleen, mesenteric lymph node and brain TIMP-1 values.

3. Results

3.1. Clinical appearance

Preclinical mice were euthanized at 100 or 160 days after intracerebral (i.c.) inoculation with scrapie-positive sheep brain. These time points were selected based on historical knowledge of the course of scrapie in Tg338 mice. At these points, infected mice were clinically indistinguishable from un-inoculated control mice. Clinical mice were euthanized when signs compatible with those reported by Campbell [7] first presented. Clinical signs consisted of severe ataxia with or without hind limb paralysis; severe lethargy compared to un-inoculated controls; and depressed response to external stimuli. Such clinical presentations were noted in the Tg338 mice infected with scrapie-positive sheep brain between 190 and 268 days post-inoculation. Mice inoculated with scrapie-negative sheep brain were clinically indistinguishable from un-inoculated mice throughout the duration of the study.

3.2. Western blots

3.2.1. Brain

Western blots were performed on pooled brain tissue of Tg338 mice that were infected with scrapie-positive sheep brain, as well as Tg338 mice infected with scrapie-negative sheep brain (Fig. 1). A three-banded pattern after proteinase-K (PK) digestion, presumably representing the diglycosylated, monoglycosylated, and nonglycosylated forms of PrP^{Sc}, confirmed the diagnosis of scrapie in those mice inoculated with scrapie-positive sheep brain. PK completely eliminates the PrP^{C} form of prion, and no band will present if no, or only very small amounts, of PrP^{Sc} are present. The three-banded pattern was noted in tissues at the clinical time point (≥190 d.p.i.) and at 160 d.p.i. The chemiluminescent signal was weaker at the 160 day time point compared to the clinical time point. No signal was present for tissues from the PK-digested 100 day time point or from mice inoculated with brain from scrapie-negative sheep that had been PK-digested.

3.2.2. Spleen

Western blots were performed on the spleens of individual mice inoculated with scrapie-positive homogenate at the 100, 160 and ≥190 d.p.i. timepoints. Three-banded patterns were noted at all time points. Signal intensity appeared most variable between individual animals at the 160 d.p.i. timepoint (Supplemental Figure).

3.3. PET blots

PET blotting, which has increased protein sensitivity compared to immunoblotting, was performed on brain tissue from individual mice infected with scrapie-positive sheep brain homogenate. Immunolabeling corresponding to the presence of PK resistant PrP^{Sc} was observed in representative brains at all 3 time points (Fig. 2). Immunolabeling intensity was greatest at the clinical time point and weakest at the first pre-clinical time point of 100 d.p.i. The 160 d.p.i. preclinical time point had intermediate stain-

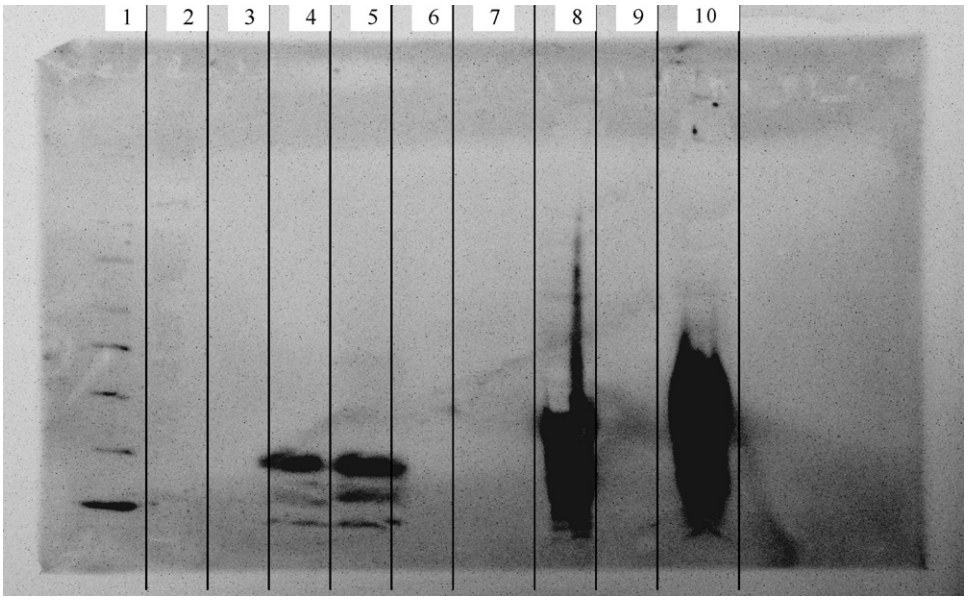

Fig. 1. Western blot on brain tissue of scrapie-infected mice. Lanes contain following: (1) MagicMark™ XP Western protein standard (Invitrogen). (2) Gel marker: MultiMark® multi-colored standard (Invitrogen). (3) Brain tissue from animal 100 days P.I., PK digestion. (4) Brain tissue from animal 160 days P.I., PK digestion. (5) Brain tissue from animal ≥190 days P.I., PK digestion. (6) Tg338 injected with scrapie-negative sheep brain (≥190 days P.I.), PK digestion. (7) EMPTY. (8) ≥190 days P.I. without PK digestion. (9) EMPTY. (10) Tg338 injected with scrapie-negative sheep brain (≥190 days P.I.), without PK digestion. In scrapie-infected mice, signal was strong at the ≥190 days P.I. (5) time point. At 160 days P.I. (4), signal was present, but less so compared to 5. No signal was observed at 100 days P.I. (3).

ing. Brain tissue from un-inoculated Tg338 and Tg338 mice inoculated with scrapie-negative homogenate presented similarly, with no detectable immunolabeling.

3.4. Histological findings

The brains of 100 d.p.i., 160 d.p.i. (both pre-clinical time points), clinical (≥190 d.p.i.), and control (inoculated with scrapie-negative homogenate) animals were examined histologically (Fig. 3). The obex of mice collected 100 days post-inoculation with scrapie-positive sheep brain showed little to no difference from the obex of control animals. The obex samples of mice that were collected 160 d.p.i. showed mild vacuolization of the gray matter. Vacuoles were of varying sizes, and in some areas involved and impinged on nearby neurons. Neu-

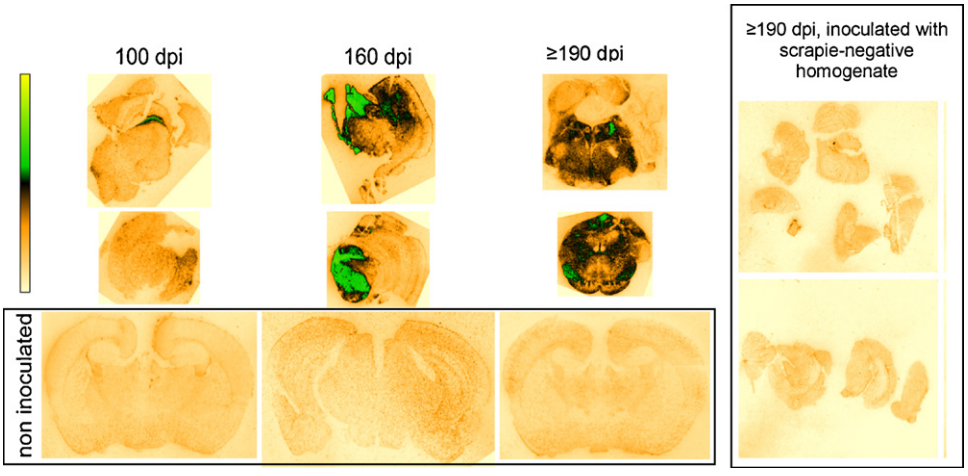

Fig. 2. PET blot immunoassay on brain tissue. PET blot immuoassay for PrP^{Sc} accumulation in the brain of mice at 100, 160 and ≥190 d.p.i with scrapie-positive sheep brain was performed. Shown are examples of PK-resistant immunoreactivities from two brain regions (obex and thalamus) of an inoculated mouse at each time point. Below each pair is an image of the corresponding membrane negative control tissue (non-inoculated mouse brain). On the far right are two images of control mice that were inoculated with scrapie-negative sheep brain homogenate and sacrificed at the corresponding clinical time point of the scrapie-positive sheep brain inoculated mice. Relative reaction differences are visualized using the ICA2 lookup table (ImageJ).

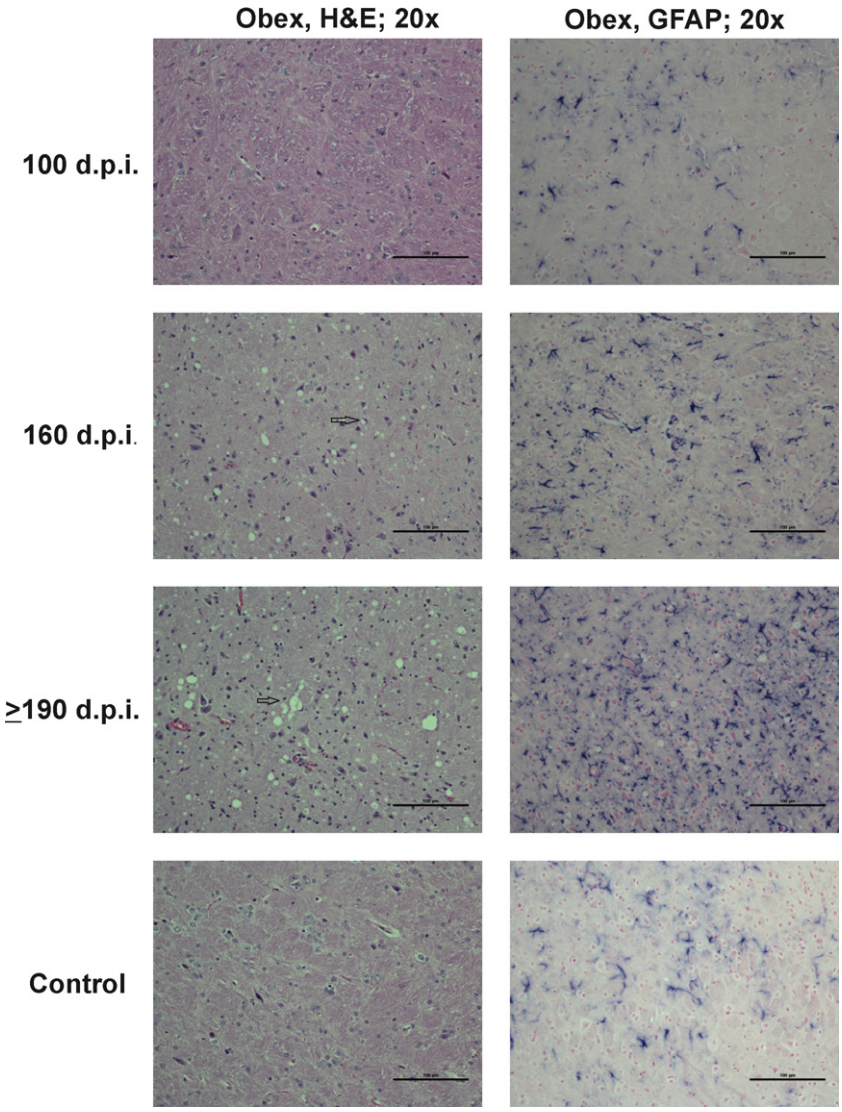

Fig. 3. Histological appearance of the obex of scrapie-injected mice (20×), with H&E and GFAP staining. Representative histological sections are shown. Obex from a 100 d.p.i. animal is unremarkable. A moderate number of neuropil vacuoles (arrow head) were noted scattered throughout the obex of the 160 d.p.i. animal. Similar vacuoles were present in the obex of a clinical animal (≥190 d.p.i.), but at a greater number, and with confluence. Few to no inflammatory cells were present in brain sections. Mild gliosis, assessed by an increased GFAP staining over control, was first noted at 160 d.p.i. At ≥190 d.p.i., this gliosis was mild-moderate in nature.

rons appeared small with compacted nuclei. Similar, but more widespread and severe, vacuolization and neuronal abnormalities were noted in the obex of mice showing clinical signs (≥190 d.p.i.). Mild gliosis was noted in the obex of infected mice at 160 d.p.i and mild to moderate gliosis was noted in mice at ≥190 d.p.i. Inflammatory cell accumulations were not recognized within vascular or extravascular tissues. The glial staining pattern was not noticeably different between the 100 d.p.i. mice and control animals.

Consistent with Bruce [22], the mice at 160 d.p.i. could be classified by histological appearance as mildly affected (mean vacuolization score = 2.2, $n = 7$) and those collected at ≥190 d.p.i. classified as severely affected (mean vacuolization score = 4.71, $n = 7$).

3.5. Cytokine assay results

Table 1 shows the cytokine patterns in 4 tissue types at 3 time points during the course of scrapie infection, with the latest time point correlating to the onset of clinical neurologic signs. After individual mouse cytokine values were normalized, expression values were averaged for each tissue type at each time point for control (Tg338 mice inoculated with scrapie-negative sheep brain) and infected (Tg338 mice inoculated with scrapie-positive sheep brain) animals. Similar to Lu [24] and Koedel [25], data were expressed as 2, 4, 6, 8 or greater-fold increases or decreases in Tg338 mice infected with scrapie-positive sheep brain over Tg338 control animals injected with scrapie-negative sheep brain.

Table 1
Protein expression in brain and periphery over course of scrapie infection in Tg338 mice.

Protein expression	Pre-clinical (100 days post infection)				Pre-clinical (160 days post infection)				Clinical (≥190 days post-infection)			
	Brain	Spleen	Mesenteric lymph node	Serum	Brain	Spleen	Mesenteric lymph node	Serum	Brain	Spleen	Mesenteric lymph node	Serum
axl	↑↑↑		↑		↑	↓		↓	↑↑↑↑		↑↑↑	
BLC			↓		↑	↓	↓		↑		↓	↑↑
CD30L			↓			↓↓	↓					↑↑
CD30/TNFRSF8	↑↑	↑	↑		↑		↓		↑	↑↑		↑
CD40	↑				↑	↓		↓	↑		↑↑↑	↑
CRG-2												↑
CTACK						↓						
CXCL16				↑		↑						↑
Eotaxin	↑↑↑	↑	↑↑	↓	↑↑			↓↓	↑	↑	↑	↓
Eotaxin-2	↑	↑	↑	↑	↑	↓↓	↓↓		↑	↑		
FAS ligand	↑						↓					
Fractal kine	↑					↓			↑↑			↑
G-CSF	↑	↑		↑	↑↑↑↑				↑↑↑	↑	↑	↑↑
GM-CSF	↑↑↑	↑	↑	↑	↑↑↑↑		↑		↑↑↑↑	↑	↑	↑↑
IFN-gamma				↑	↑↑↑				↑			↑↑
IGF-BP-3						↓	↓					
IGF-BP-5	↑		↑			↓			↑			
IGF-BP-6	↑↑			↑↑↑			↓		↑↑↑↑	↑	↑↑	↑↑↑↑
IL1-alpha						↓	↓	↓				↑
IL1-beta	↑↑						↓↓		↑	↓	↓	
IL2				↑	↑↑				↑		↓	↑
IL3		↑			↑	↓			↑	↑↑		↑
IL3Rb			↓			↓	↓			↓	↓	
IL4	↑	↑	↑↑	↑	↑↑		↑		↑	↑	↑	↑↑
IL5	↑↑↑	↑↑	↑↑	↑	↑↑↑↑	↑	↑↑		↑↑↑	↑	↑	↑↑
IL6	↑↑↑↑	↑↑↑	↑↑↑	↑↑	↑↑↑↑		↑		↑↑↑↑	↑↑	↑	↑↑↑
IL9	↑	↑	↑	↑	↑				↑	↑	↑	↑↑↑
IL10	↑	↑	↑	↑	↑↑			↑	↑			↑↑↑↑
IL12-p40/p70	↑↑		↑	↑	↑↑↑				↑		↑↑	↑
IL12-p70	↑		↑	↑	↑							↑
IL13	↑					↓↓↓↓	↓		↑			↑
IL17	↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓	↑				↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
KC					↑		↓		↑			↑
Leptin R	↑					↓			↑	↑↑	↑↑	↑
Leptin	↑↑↑	↑↑	↑↑		↑			↓↓	↑↑	↑↑	↑	
LIX				↑↑↑↑		↑				↑↑		↑↑↑↑
L-Selectin						↓	↓↓	↓		↑	↑↑↑	
Lymphotactin				↑		↓				↑		↑
MCP-1	↑			↑	↑↑↑↑				↑			↑
MCP-5			↓			↓↓↓↓	↓↓	↓				
M-CSF						↓↓	↓↓↓	↓				↑
MIG	↑↑↑↑	↑↑↑↑	↑↑		↑↑			↓↓↓	↑↑↑	↑↑	↑	
MIP-1-alpha		↓	↓			↓↓↓↓	↓↓↓					↑
MIP-1-gamma			↑↑				↑	↓	↑		↑↑↑↑	
MIP-2			↑							↑		↑
MIP-3-beta					↓	↓↓↓	↓↓					
MIP-3-alpha			↓			↓↓↓	↓	↓				↑
PF4	↑↑	↑	↑↑↑	↑					↑↑	↑↑↑	↑	↑
P-Selectin			↑						↑		↑↑	
RANTES		↓									↑↑↑↑	
SCF	↑		↑		↑	↓		↓↓				
SDF-1 alpha	↑	↑↑		↑↑↑↑	↑↑		↑		↑↑	↑		↑
TARC						↓	↓	↓				
TCA-3			↓		↓	↓	↓↓	↓			↓	
TECK			↓			↓		↓	↑			↑
TIMP-1	↑↑↑	↑↑↑↑	↑↑	↑↑↑↑	↑↑↑↑	↑	↑	↑↑↑	↑↑	↑↑↑	↑↑	↑↑↑↑
TNF-alpha	↑	↑	↑	↑↑	↑↑		↑		↑	↑	↑	↑↑↑
sTNF Rl	↑↑↑↑	↑	↑↑↑		↑		↑		↑↑↑↑	↑↑	↑↑↑↑	↑
sTNF Rll	↑↑	↑	↑		↑↑↑↑		↑	↓	↑↑↑↑	↑↑↑	↑↑↑↑	
TPO				↑					↑			↑
VCAM-1	↑↑		↑↑		↑		↑		↑↑		↑↑↑↑	
VEGF	↑	↑↑	↑					↓↓↓	↑	↑↑		

↑2 times greater than controls; ↑↑4 times greater than controls, ↑↑↑6 times greater than control, ↑↑↑↑8+ times greater than controls↓2 times less than controls; ↓↓4 times less than controls, ↓↓↓6 times less than control, ↓↓↓↓8+ times less than controls. Expression of cytokine and cytokine-related proteins in brain, spleen, mesenteric lymph nodes and serum at two pre-clinical and one clinical time point during infection. Values are expressed as 2, 4, 6 or ≥8 times greater (↑) or less (↓) than controls. Control mice are Tg338 animals injected with scrapie-negative sheep brain.

In the spleen, mesenteric lymph nodes and serum, cytokine expression levels were high at the earliest and latest time points, with a transient decline in expression levels at the intermediate time point. In the spleen, 20 of 62 cytokine or cytokine-related proteins were increased at least 2 times greater than controls at 100 d.p.i., 4 of 62 cytokine or cytokine-related proteins were increased at least 2 times greater than controls at 160 d.p.i., and 25 of 62 cytokine or cytokine-related proteins were increased at least 2 times greater than controls at ≥190 d.p.i. At 100 d.p.i., expression levels for 3 cytokine proteins in the spleen were at least two times lower than controls, 24 cytokines were at least two times lower than controls at 160 d.p.i., and 3 cytokine proteins were at least two times lower than controls at ≥190 d.p.i. In the mesenteric lymph nodes, 26 of 62 cytokine or cytokine-related proteins were increased at least 2 times greater than controls at 100 d.p.i. and 9 cytokine proteins were at least two times lower, 11 of 62 cytokine or cytokine-related proteins were increased at least 2 times greater than controls at 160 d.p.i. and 20 were at least 2 times lower. 24 of 62 cytokine or cytokine-related proteins were increased at least 2 times greater than controls at ≥190 d.p.i. in the mesenteric lymph nodes, and 6 were at least two times lower. The serum had increases of 22/62, 2/62 and 38/62 proteins at 100, 160, and ≥190 d.p.i., respectively; decreases of at least two times compared to controls were 3/62, 18/62 and 2/62. The cytokines that were expressed in the spleen, mesenteric lymph nodes and serum at 160 d.p.i. were generally lower than those at the 100 d.p.i. and ≥190 d.p.i., primarily only 2 times above expression levels of controls.

At all time points studied, the brain was the tissue type that had the largest number of cytokines expressed. Cytokine expression increases were consistent across all three time-points in the brain. Thirty-three of 62 cytokine or cytokine-related proteins were increased at least 2 times greater than controls at 100 d.p.i, 30/62 increased at least 2 times greater than controls at 160 d.p.i, and 39/62 increased at least 2 times greater than controls at ≥190 d.p.i. There were few cytokines that had expression levels less than that of controls in brain tissue: one at 100 d.p.i., two at 160 d.p.i. and one at ≥190 d.p.i. IL-17 was the lone cytokine decreased below control levels at the earliest and latest time points in the brain.

TIMP-1 (TIMP metallopeptidase inhibitor 1) was the only cytokine to be expressed in all tissue types at all time points at values of at least 2 times greater than controls. The presence of TIMP-1 in the serum at 160 d.p.i. at levels that were 6 times greater than controls is noteworthy considering the lack of increased cytokine expression overall in the 160 d.p.i. animals, especially in the serum. Interleukin-5 and 10 (IL-5, IL-10, respectively) should also be noted for their expression profiles throughout infection. Both of these cytokines had expression in 3 of 4 tissue types at the 160 d.p.i. time point. Since IL-10 and TIMP-1 were the only cytokines expressed in the serum at all time points, statistical testing was carried out to determine if such expression was statistically significant over controls.

The decreased expression of IL-17 is striking. Expression is at least 4 times, and more often 8+ times, less than controls in all tissues at the 100 and ≥190 d.p.i. time points, while at 160 d.p.i., expression of this cytokine protein is similar to that of control animals.

3.6. Statistical analysis

The difference for normalized serum values between infected and control mice for TIMP-1 was significant at 160 d.p.i ($p = 0.018$). The increase over controls was also significant for serum values at 100 d.p.i. ($p = 0.001$) and ≥190 d.p.i. ($p = 0.001$). Similarly, serum values for IL-10 were significantly increased over controls for all time points: $p = 0.008$ at 100 d.p.i., $p = 0.043$ at 160 d.p.i., and $p = 0.016$ at ≥190 d.p.i. (Fig. 4). Additional statistical testing for TIMP-1 brain and spleen values demonstrated a significant increase over controls at 100 and ≥190 d.p.i. for both tissue types (brain: $p = 0.008$ at 100 d.p.i. and $p = 0.035$ at ≥190 d.p.i.; spleen: $p = 0.004$ at 100 d.p.i. and $p = 0.016$ d.p.i. at ≥190 d.p.i.). TIMP-1 and IL-10 values at 160 d.p.i. for spleen and brain were not significantly increased over controls, nor was

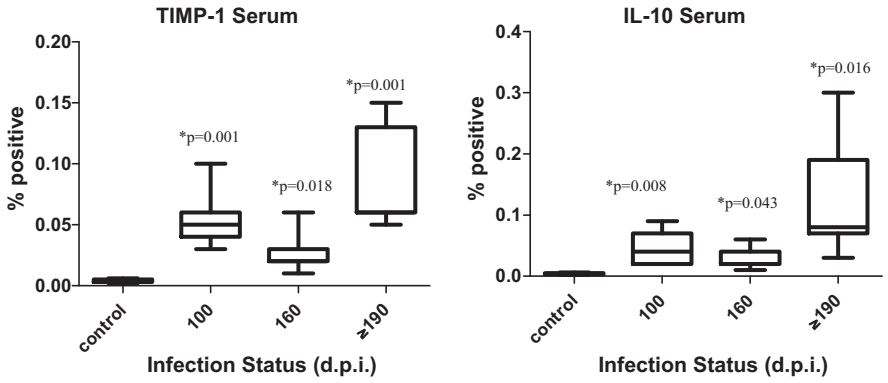

Fig. 4. TIMP-1 and IL-10 expression in serum of scrapie-infected mice compared to controls. For TIMP-1 and IL-10, animals at all times post-infection had significantly different (*) cytokine levels compared to controls in serum samples. Unpaired *t*-tests were performed, with Welch's correction used for those groups with unequal variances. Significance was determined at $p < 0.05$. Box plot whiskers represent the minimal and maximal observations for each group of data, with the line indicating the median value and the central box spanning the 25th to 75th quartiles of data. Cytokine values were normalized as % positive. Controls are Tg338 mice inoculated with scrapie-negative sheep brain that were collected at points equivalent to the clinical mice inoculated with scrapie-positive sheep brain.

expression in the mesenteric lymph nodes at any of the three time points (data not shown).

4. Discussion

The appearance of clinical signs and histological evaluation of brain tissue are the most common means of diagnosing scrapie in a sheep herd. Similar to other prion diseases, clinical signs of scrapie infection do not present until the end stage of infection. Histological changes in the brain also occur late and lack a robust inflammatory cell component. Work using mRNA as a determinant suggests that, at least at the end-stage of infection in the brain, cytokine secretion is amplified [7–9]. Identifying certain key cytokines and evaluating their expression dynamics throughout the entire course of disease could provide useful biomarkers of infection. In this regard, an individual or group of cytokines present in the serum throughout infection could be particularly useful for ante-mortem diagnosis or tracking of disease. Similarly, knowledge of the tissue expression profile and kinetics of such proteins could contribute valuable information to the pathogenesis of this still somewhat elusive group of prion diseases. This study sought to determine if cytokine protein expression patterns developed during scrapie infection (as suggested by mRNA data), and if so, what those patterns were in brain and in a variety of peripheral tissues at several time points of infection-prior to and at the on-set of clinical signs, as well as prior to and once histological brain changes were noted.

The clinical characteristics of scrapie disease at the end point of infection are described in several murine models. Using the Tg338 mouse strain with, relative to other models, a historically reliable incubation time allows preclinical time points to be studied with some degree of consistency from animal to animal. The Tg338 mouse expresses sheep, but not mouse, PrP^{c} and is susceptible to scrapie infection after inoculation with brain homogenates derived from sheep naturally infected with scrapie. Hence, the murine PRNP knock-out background strain of the Tg338 model eliminates the interfering effect of endogenous mouse PrP^{c}. In contrast to the Tg338 strain, other murine models of scrapie must use mouse-adapted scrapie strains, such as ME7 or RML, which have been repeatedly passaged in mice in order to overcome the species barrier to infection [26]. Tg338 mice reliably show clinical signs between 6 and 9 months post-infection [12,13], and in this study, animals showed signs between 190 and 268 d.p.i. (6.3 and 8.9 months respectively).

Significant change in the expression profile of cytokines and cytokine-like proteins was detected in this study in preclinical mice at 100 d.p.i. Detection of PrP^{Sc} accumulation in the brain by sensitive PET blot analysis, as well as detectable signals on splenic tissue western blots, confirms these scrapie-positive sheep brain inoculated mice were indeed incubating disease, although in addition to not showing clinical signs of disease, histologically, the 100 d.p.i. animals appeared similar to controls. At 100 d.p.i., increased expression levels for most cytokines were as much as, or even more than, those detected at the end point of infection during clinical disease.

For many of the cytokines examined in the spleen, mesenteric lymph nodes and serum, expression levels at the intermediate time point of 160 d.p.i. were decreased compared to values noted earlier and later in infection, with some protein levels dipping below that of control animals. At the 160 d.p.i., animals were still not showing signs of clinical disease, but vacuolization and gliosis were noted on histological evaluation of brain tissue. Similar to our findings, a "dip" in mRNA cytokine expression mid-infection was reported by Lu [24] in their study of Creutzfeldt-Jakob disease (CJD). This biphasic inflammatory response was presumed to be the result of infectious agent replication initially inducing then suppressing host transcriptional responses. Waxing and waning cytokine levels throughout infection have also been reported by Romero-Trevejo [27] in various intestinal cell populations of scrapie-infected mice.

Brain cytokine protein expression in the current study was fairly consistent with 23/62 cytokines expressed in brain tissue at all time points tested. The inflammatory response in prion-infected spleens has been deemed virtually undetectable in other studies of prion infection compared to the activation of glia in the CNS [10,28]. Our study shows that significant changes in cytokine expression in the spleen and lymph nodes are present, though dynamically so, during the course of infection. At all time points in the mesenteric lymph nodes, ten cytokines had increased expression. Besides the brain, the mesenteric lymph nodes had the most consistent expression pattern throughout infection.

Protein expression of 24 cytokines just at the end stage of infection and only in the brain of scrapie-infected animals was recently studied and reported by Tribouillard-Tanvier [29]. Wildtype and transgenic mice expressing anchorless mouse PrP were infected with the 22L mouse scrapie strain. Levels of 10/24 cytokines assayed in brain tissue were increased. Nine of these 10 increased cytokines were measured in our study (IL-1α, IL-1β, IL-6, GM-CSF, IFN-γ, RANTES, MCP-1, KC and IL-12p40). We found that protein levels for 7 of the 9 (all but IL-1α and RANTES) were noted to be expressed in brain tissue at least 2 times over control animals at the end stage of infection. We also found 7 of the 9 (all but IL-1β and RANTES) to have increased protein expression over controls in the serum at the end stage of infection. Tribouillard-Tanvier [29] commented that compared to bacterial or viral infections, the levels of cytokines in scrapie-infected brains were low and somewhat disproportionate to the extensive gliosis and astrogliosis observed. Similar remarks have been made in response to the cytokine gene expression in ME7 mouse models of scrapie [30]. A possible explanation for any differences between cytokine profiles in our study compared to others may be attributable to the animal model used. Tg338 animals over-express PrP^{c} in the brain, which could possibly make subtle differences in the expression of some cytokines more obvious or alter the significance of others. Also, infection in the Tg338 strain was initiated directly with scrapie-positive sheep homogenate rather than mouse-passaged homogenate.

By the time clinical signs were present, 38/62 cytokines examined in this study had at least 2 times greater expression in the serum of infected mice over control mice, although only 2 of the cytokines studied, TIMP-1 and IL-10, were consistently expressed in the serum at least 2 times greater than controls at all time points studied. This increase was significant for TIMP-1 and IL-10 at all time points. These cytokines in particular may represent markers or patterns of markers useful in detecting scrapie in preclinical animals by antemortem analysis.

TIMP-1 was the only cytokine expressed in all tissues at all time points. TIMPs are endogenous tissue inhibitors of matrix metalloproteinases (MMPs). TIMPs and MMPs are tissue remodeling proteins and inflammatory mediators that have been indicated in neurologic conditions such as Alzheimer's [31]. TIMP-1 production has been associated with injured neurons and its activity is associated with astroglial reactivity [32]. MMP and TIMP expression patterns in microglia have been implicated as determining the cytokine production of activated microglial cells following CNS injuries [31]. Increased TIMP-1 levels have been noted in the serum of human patients with amyotrophic lateral sclerosis (ALS), multiple sclerosis, Guillain-Barré syndrome and viral or bacterial meningitis [33].

The significance of the decreased levels of IL-17 in all tissues at early and late time points, but not during mid-infection, is unknown. IL-17 is a pro-inflammatory cytokine most-often thought to be secreted by helper T-cells and microglia, although other cells involved in innate and adaptive immunity, especially those involved in autoimmunity associated with multiple sclerosis, have been implicated in secretion of this cytokine [34,35]. Similar to our findings, IL-17 was assessed in the study by Tribouillard-Tanvier and reported as one of the cytokines that was not elevated or detected in measurable amounts in scrapie positive brain tissue [29].

In conclusion, this study focused on screening ovinized transgenic mice for cytokine and cytokine-related protein expression at preclinical and clinical time points of scrapie infection. Cytokine levels were increased in all tissues studied early in infection, suggesting that a complex host-response was induced in peripheral tissues and brain of infected animals well before clinical signs or histologic brain changes were apparent. As infection progressed, the cytokine response was dynamic, most notably in sera and peripheral tissues. Identification of an on-going, dynamic cytokine response in tissues other than the brain is informative for studies of comparative pathogenesis and for the potential development of future preclinical diagnostic indicators.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cimid.2011.06.001.

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